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The SPI-19 encoded type-six secretion-systems (T6SS) of *Salmonella enterica* serovars Gallinarum and Dublin play different roles during infection

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ABSTRACT

Salmonella Pathogenicity Islands 19 (SPI19) encodes a type VI secretion system (T6SS). SPI19 is only present in few serovars of *S. enterica,* including the host-adapted serovar *S.* Dublin and the host-specific serovar *S.* Gallinarum. The role of the SPI19 encoded T6SS in virulence in these serovar is not fully understood. Here we show that during infection of mice, a SPI19/T6SS deleted strain of *S.* Dublin 2229 was less virulent than the wild type strain after oral challenge, but not after IP challenge. The mutant strain also competed significantly poorer than the wild type strain when co-cultured with strains of *E. coli,* suggesting that this T6SS plays a role in pathogenicity by killing competing bacteria in the intestine. No significant difference was found between wild type *S.* Gallinarum G9 and its ΔSPI19/T6SS mutant in infection, whether chicken were challenged orally or by the IP route, and the *S.* Gallinarum G9 ΔSPI19/T6SS strain competed equally well as the wild type strain against strains of *E. coli.* However, contrary to what was observed with *S.* Dublin, the wild type G9 strains was significantly more cytotoxic to monocyte derived primary macrophages from hens than the mutant, suggesting that SPI19/T6SS in *S.* Gallinarum mediates killing of eukaryotic cells. The lack of significant importance of SPI19/T6SS after oral and systemic challenge of chicken was confirmed by knocking out SPI19 in a second strain, J91. Together the results suggest that the T6SS encoded from SPI19 have different roles in the two serovars and that it is a virulence-factor after oral challenge of mice in *S.* Dublin, while we cannot confirm previous results that SPI19/T6SS influence virulence significantly in *S.* Gallinarum.

1. Introduction

The Gram-negative bacterium, *Salmonella enterica,* contain more than 2600 serovars (Issenhuth-Jeanjean et al., 2014), some of which have a narrow host range, while most, such as *S.* Typhimurium, are capable of infecting a wide range of mammalian and avian species. A distinct feature of the host adapted/specific serovars is an ability to cause a systemic, typhoid-like infection in their preferred host (Uzzau et al., 2000). *S.* Dublin infects cattle but can occasionally infect other mammals including humans and mice (Uzzau et al., 2000), while *S.* Gallinarum is specific to avian hosts and is considered non-pathogenic to man (Chadfield et al., 2003).

Type 6 secretion systems (T6SS) are wide spread in Gram negative bacteria (Pukatzki et al., 2006), and a recent review estimated that such

systems are present in up to a quarter of all Gram negative species (Basler, 2015). They are evolutionary related to contractile elements of phages and work as energy driven spikes that can enter the cytoplasm of other bacterial cells or eukaryotic cells (Basler, 2015). Often, they deliver proteins which are cytotoxic (Ray et al., 2017). T6SSs have been characterized as virulence factors in several pathogens, including *Legionella pneumophila* (Sexton et al., 2004; Zink et al., 2002), *Vibrio cholera* (MacIntyre et al., 2010; Pukatzki et al., 2007), *Burkholderia mallei* (Schell et al., 2007), avian pathogenic *Escherichia coli* (de Pace et al., 2011, 2010), *Edwardsiella tarda* (Rao et al., 2004; Zheng and Leung, 2007), *Pseudomonas aeruginosa* (Mougous et al., 2006), *Yersinia pestis* (Andersson et al., 2017), *Bordetella bronchiseptica* (Bendor et al., 2015) and *Salmonella* (Blondel et al., 2013, 2010; Pezoa et al., 2013; Sana et al., 2016).

Only a limited number of *Salmonella* serovars carry T6SSs. The first described T6SS in *Salmonella* was the one encoded in *Salmonella* pathogenicity island-6 (SPI6) in *S.* Typhimurium; this gene island was formerly known as *S. enterica* centisome 7 island (Folkesson et al., 2002). Blondel et al., (Blondel et al., 2009) searched through available genomes and detected gene-islands (SPI19-SPI21) encoding novel T6SSs. SPI19 was found in *S.* Dublin, *S.* Weltevreden, *S.* Agona, *S.* Gallinarum and *S.* Enteritidis, though in *S.* Enteritidis, it was only present in a truncated version. *S.* Dublin was found to carry two T6SS (Blondel et al., 2009), of which only the T6SS encoded in SPI6 was reported to be a virulence factor (Pezoa et al., 2013). The SPI6 encoded T6SS in *S.* Typhimurium was first described as a survival factor inside macrophages (Mulder et al., 2012), but more recent studies could not confirm the role in systemic infection and showed that the system constitutes an anti-bacterial weapon in the intestine. The wild type *S.* Typhimurium SL1344 strain reached significantly higher counts in feces of mice than a T6SS-deletion mutant, and the role of the T6SS was shown to be during early colonization where it killed other microbiota members (Sana et al., 2016).

S. Typhimurium is considered an intestinal pathogen, and T6SS can be viewed as yet another weapon used by this serovar to create a growth advantage in the intestine (see (Herrero-Fresno and Olsen, 2018), for a recent review on these strategies). In contrast to *S.* Typhimurium, the narrow host-range serovars, such as *S.* Dublin and *S.* Gallinarum, are not believed to depend entirely on propagation in the intestine for long term survival (Uzzau et al., 2000). They may still be faced with competition in the intestine, but it may also be that the T6SS plays a role in virulence of these bacteria during propagation at systemic sites. The aim of the current study was to revisit the SPI19 encoded T6SS of *S.* Dublin and *S.* Gallinarum to further investigate their potential role in virulence.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strains and plasmids used are listed in Table 1. Strains consisted of wild type and ΔSPI19/T6SS mutants of *S.* Dublin 2229 and *S.* Gallinarum G9 and J91. *S.* Typhimurium 4/74 and *S.* Typhimurium 4/74 Δ*invH* were included as a control in some experiments, and commensal *E. coli* strains from the strain collection of Department of Veterinary and Animal Sciences, University of Copenhagen, were used in competitive growth experiments. Lennox broth (LB) medium and LA-agar (Becton, Dickinson and Company) was used for culturing of bacteria. M9 medium (Miller, 1972) supplemented with 0.2% glucose, thiamine (1 μg/ml), cysteine, leucine and aspartic acid (100 μg/mL) was used to compare the ability of mutants and wildtype strains to grow in minimal medium. When needed, antibiotics (Sigma) were supplemented in the following concentrations: Kanamycin (Kan) 50 μg/ml, ampicillin (Amp) 100 μg/ml, chloramphenicol (Cam) 15 μg/ml, rifampicin (Rif) 50 μg/ml and tetracycline (Tet) 10 μg/ml.

2.2. DNA isolation and purification

DNA for use as PCR template was obtained with DNeasy Blood & Tissue Kit (Qiagen). PCR products used for cloning were purified with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Life Sciences). Plasmids were purified with Qiagen Mini Kit or Qiagen Midi Kit. All kits were used according to the manufacturers' instructions.

2.3. Construction of mutants

Construction of mutants was first performed by insertion of cassettes encoding antibiotic resistance (Kan and Cam) into relevant genes by the Lambda Red recombinase system (Datsenko and Wanner, 2000). Insertions were confirmed by PCR. The knock-out in *S.* Gallinarum and *S.* Dublin obtained by this method was internal to the full T6SS and corresponded to the region that is not present in *S.* Enteritidis. It spanned the genes SG1031-SG1048 and SeDA1218-SeDA1235. All primers used for construction of PCR products and for control of insertion in the correct manner are listed in Table 2.

Two further deletion mutants of *S.* Gallinarum, one in strain G9 and one in strain J91, were constructed by allelic exchange. These mutations spanned the full SPI19 region. The *cat*-gene from pTSC29

Table 1
Strains and plasmids used in this study.

Strains	Description	References
S. Dublin 2229	Wild type strain	(Baird et al., 1985)
S. Dublin 2229 ΔSPI19/T6SS	*SeD_A1218-SeD_A1235::kan.* Kan[R].	This study
S. Dublin 2229 FRT-SPI19/T6SS	*SeD_A1236::scar SeD_A1217::kan.* Kan[R].	This study
S. Gallinarum G9	Wild type strain	(Barrow et al., 1994)
S. Gallinarum G9[rif]	G9-rifampicin resistance	This study
S. Gallinarum G9 ΔSPI19/T6SS	SG1031-SG1048::kan. Kan[R].	This study
S. Gallinarum G9 ΔSPI19	Kan[R]	This study
S. Galllinarum J91	Wild type strain	(Christensen et al., 1993)
S. Gallinarum J91 ΔSPI19	KanR	This study
S. Typhimurium 4/74	Wild type strain	(Jones et al., 1988)
S. Typhimurium 4/74 Δ*invH*	*invH*::Kan. Kan[R]	(Herrero-Fresno et al., 2018)
E. coli DH5α	Host for pSLD45FT.	
E. coli V517	Plasmid marker.	(Macrina et al., 1978)
E. coli 39R861	Plasmid marker.	(Threlfall et al., 1986)
E. coli 5028	WT commensal from layer chicken (Eco_1)	Strain collection IVH[#]
E. coli 5029	WT commensal from layer chicken (Eco_2)	Strain collection IVH[#]
E. coli 907	WT commensal from calf (Eco_3)	Strain collection IVH[#]
E. coli 901	WT commensal from calf (Eco_4)	Strain collection IVH[#]
Plasmids		
pKD4	The kanamycin resistance (Kan[R]) was used as marker in mutations. Kan[R].	(Datsenko and Wanner, 2000)
pKD46	Heat sensitive. Arabinose inducible Lambda Red recombinase system. Amp[R].	(Datsenko and Wanner, 2000)
pCP20	The plasmid encodes FLP, used for excision at FRT sites. Amp[R], Cam[R].	(Datsenko and Wanner, 2000)
pSLD45FT	Amp[R]. Modified version of pCTX-M3 (Gniadkowski et al., 1998; Golebiewski et al., 2007)	This study
pTSC29	Temperature sensitive plasmid	(Phillips, 1999)
pCAS1	Amp[R].	This study.
pDubSPI19-1	Complementation plasmid, Amp[R], Kan[R],	This study.

IVH: Department of Veterinary and Animal Sciences, University of Copenhagen.

Table 2
List of primers used for construction of mutants, for control of the correct insertion of antibiotic gene cassettes for interruption of genes and for qPCR analysis.

Primer	Sequence 5' → 3'	Use of primer
CAS9	TTCTGTACGCCAGTCGTCATTTTCATGGGCATGACGGGTATGTGTAGGCTGGAGCTGCTTC	Construction of antibiotic resistance cassette
CAS10	TGGATCTGTACCTGTGACAGGGTTCAGCGGATACTGGTACCATATGAATATCCTCCTTAG	As CAS9
CAS15	CAGGGAGGAGGTGAGGTACA	Mutant verification
CAS16	CCATACCTTTCCTCGGTCCT	As CAS15.
CAS17	TGTAATAAGTGGCCGTGTGC	Mutant verification
CAS19	GCCAAGCAATAACTCCTCCA	Mutant verification
CAS29	AGAAAAAGGTTCTGCATGTATTAAAGATGTAGTTAAGTCCTGTGTAGGCTGGAGCTGCTTC	Construction of antibiotic resistance cassette with two FRT sites
CAS30	AATGCCAAATTTGTTGGATCTCTTTCTCCGTCTTTTATCAGGATCCCATATGAATATCCTCCTTAG	As CAS29.
CAS34	CAAGAATTGCTGCAACGAAA	Mutant verification
CAS53	GTTCAGGAGGGGACATTCAG	Mutant verification
CAS54	GAGCCAGTCCTGCTAAAACG	Mutant verification
CAS55	TAATCACCGGAATGGTGGAT	As CAS53 and CAS54
CAS58	GTGAGACGATTTGTTGATCTCTTCCCGCAGGGCGCTGAACTGTGTAGGCTGGAGCTGCTTC	Construction of antibiotic resistance cassette with a single FRT site
CAS59	TGAGTCAGGACGCACTCACGCACATTATTGTTACCGGCCAGAAATCTCGTGATGGCAGGT	As CAS58.
168273f-Xho1	AGTACGAATTCGCCATTGGTCTTGATTATCC	Mutant construction
168709r-EcoRI	AGTACGAATTCGCCATTGGTCTTGATTATCC	Mutant construction
192948f-BaHI	AGTCTGGATCCAGGCCATGCTAATCAATAGC	Mutant construction
193480r-SacI	ACAGTGAGCTCGTGTTCGCCGTTATGAGATAG	Mutant construction
168222f	TCTGTCATCCTGGTGCCTTG	Verification of mutant construction
193553 r	TCTGCAAAGTTACCGATAGCC	Verification of mutant construction
Cat3335f-EcoRI	ACGATGAATTCTTTCCAGTCTGACCACTTCG	Construction of cat-cassette
Cat4374r-BaHI	CTGGATCCCATTCAAATATGTATCCGCTCA	Construction of cat-cassette
Cat3451r	GCGTCAGATCCGACAGTAAG	Verification of mutant construction
Cat4240f	CAACAGTACTGCGATGAGTGG	Verification of mutant construction
clpV-F	GCGATACCGATATTATGAC	qPCR
clpV-R	GCTTCTCAAATTCACCTT	qPCR
vgrG-F	CGCAGAAGAATATGAATA	qPCR
vgrG-R	GGATTTGGTTATTGGTAA	qPCR
16 s-F	TTAGATACCCTGGTAGTCCACGC	qPCR[a]
16 s-R	TTGCGGGACTTAACCCAAC	qPCR[a]

a: primer sequences for 16 sRNA gene-amplification were according to Zhai et al. (2018). Other primer sequences were designed as part of the current study.

(Phillips, 1999), including its promoter and terminator, was amplified by PCR using primers Cat3335f-EcoRI and Cat4374r-BaHI (Table 2), and cloned using the TOPO TA cloning® kit as described by the manufacturer (Invitrogen). Transformants were selected on LB-agar (LA) with 20 μg/mL Kan. Plasmids were purified, and the *cat*-cassette was isolated from the rest of the plasmid by digesting with EcoRI and BamHI followed by gel-purification with a GFX-column (Amersham). The primer pairs 168273f-Xho1/168709r-EcoRI and 192948f-BaHI/193480r-SacI (Table 2) were used to amplify 517 bp and 533 bp fragment flanking the intended knock-out region in *S.* Gallinarum G9 chromosome. PCR products were purified with microspin columns S-400 (Amersham biosciences), digested with Xho1 and EcoR1 and Sac1and BamH1, respectively and cloned into Xho1- and Sac1-digested, temperature sensitive plasmid PTSA29 (Phillips, 1999). Transformation of ElectroMAXTM DH10BTM cells (Invitrogen) and selection of Kat resistant colonies was performed as described by the manufacturer. The correct size of the insert was verified by digesting the plasmid with XhoI and SacI. The restriction and modification negative strain *S.* Typhimurium KP1274 was electroporated with the plasmid and from there, plasmids from two transformants were purified and electroporated into competent *S.* Gallinarum G9 with selectin on LA-agar with 20 μg/mL Kan and incubation at the non-permissive temperature 42 °C. To promote a second recombination event (outcross), the integrants were grown in LB at 30 °C for 6 h. The culture was serially diluted and plated on LA-agar with Kan and streaked again under the same conditions. The presence of an insert was verified with primer pairs 168222f/Cat3451 r and Cat4240f/193553 r (Table 2). Curing of the plasmid was done by growing 42 °C in LB with 5 μg/mL Kan to stationary phase and sub cultured (diluting 1:100) repeatedly. The culture was tenfold serially diluted and plated on both LA with Am and LA with Kan and incubated at 37 °C. A number of Kan colonies were tested for Am-sensitivity. The chromosomal deletion of these was verified by PCR with primers

168222f and 193553 r (Table 2). The deletion was transferred to S. Gallinarum J91 by P22 transduction as described (Maloy, 1996).

2.4. Complementation of SPI19 in trans

2.4.1. Preparation of a suitable vector for insertion of large DNA sequences

To be able to complement the deletion of the 25–27 kb large SPI19 fragment of *S.* Dublin *in trans*, the plasmid pSLD45FT was constructed (Supplementary material, Figure S1). The plasmid was modified from the conjugative plasmid pCTX-M3 (Gniadkowski et al., 1998; Golebiewski et al., 2007). It harbours a Tet-resistance cassette flanked by FRT sites on each side, suitable for insertion of SPI19. The tetracycline resistance cassette was removed from pSLD45FT by FLP-mediated recombination (Datsenko and Wanner, 2000), leaving a scare with a single FRT site, resulting in the plasmid pCAS1 (Supplementary material, Figure S1 - top). Removal of the Tet resistance cassette was verified by PCR using primers listed in Table 2.

2.4.2. Preparation of S. Dublin 2229 SPI19 for vector insertion, and

Using the primers CAS29 and CAS30 (see Table 2) and pKD4 as template, a Kan-resistance cassette was constructed. By use of the Lambda Red recombination system (Datsenko and Wanner, 2000) the cassette was inserted in the beginning of gene SeD_A1236 in *S.* Dublin 2229, flanking SPI19. After removing the resistance cassette by introducing pCP20, a single FRT site was left next to the SPI19 sequence. On the other side of *S.* Dublin SPI19, another cassette was inserted in gene SeD_A1217. This kanamycin resistance cassette only had a single FRT site on the side turning away from SPI19. The cassette was constructed with the two primers CAS58 and CAS59 (Table 2) and pKD4 as template. Correct insertions, resulting in *S.* Dublin FRT-SPI19, were verified by PCR with the primer pairs CAS19/CAS34 and CAS17/CAS53 listed in Table 2. The plasmid pCAS1 was conjugated from *E. coli* DH5α

to *S.* Dublin 2229 FRT-SPI19, followed by transformation of pCP20 to the same strain and growth at 30 °C. Correct insertion of SPI19 was confirmed by PCR of overlapping regions with primer pairs CAS53/ CAS55 and CAS34/CAS54 (Table 2), followed by sequencing of the PCR products (Macrogen). The resulting plasmid, pSduSPI19-1, is depicted in Supplementary material, Figure S1.

2.5. Conjugation of pSDuSPI19-1 and pCAS1plasmids into S. Dublin and S. Gallinarum

Conjugation of plasmids into strain of *Salmonella* was performed on agar plates as described (Andoh et al., 2017). To select for the different recipient strains, colony size and colony morphology on XLD plates (Difco) were used. To confirm strain identity, they were tested for motility, indole formation and plasmid profiles following the procedure of Kado and Liu (Kado and Liu, 1981). The plasmid profiles of *E. coli* strains V517 (Macrina et al., 1978) and 39R861 (Threlfall et al., 1986) were used as size markers.

2.6. Growth experiments

The ability of mutant strains to grow, relative to the growth performance of the wild type strains, was measured 100 ml flasks. The strains were first grown to stationary phase in LB without antibiotics and diluted to 25 ml LB or M9 with an OD600 nm of 0.05. The flask was then further incubated in a water bath at 37 °C or 41 °C with shaking (200 rpm) for 24 h. OD600 nm was measured every 30–45 minutes. For testing of the role of T6SS in killing of other bacteria, growth assays were performed as described (Sana et al., 2016), however with modification of the media. Briefly, wild type and ΔSPI19/T6SS mutants were grown overnight at 37 °C, spun down, re-suspended in PBS and mixed 50:50 in spots on McConkey agar (Oxoid) with commensal *E. coli* isolated from healthy poultry or calves (Table 1). The T6SS in *Salmonella* is reported to be induced by bile (Sana et al., 2016), and McConkey agar contains bile salts. It was ensured by CFU counts that the inoculum of the *Salmonella* and *E. coli* strains were not significantly different (data not shown), and after overnight incubation at 37 °C, CFU of *Salmonella* and the co-cultured *E. coli* were determined by plating of serial dilutions on McConkey agar. As control experiment, all strains were seeded as described, but without co-seeding with other strains and CFU after overnight incubation at 37 °C were determined.

2.7. Expression of genes encoded from the SPI19 encoded T6SS

Two core genes of the T6SS, *clpV* and *vgrG*, were selected for qRT-PCR analysis using 16 s RNA encoding genes as internal control. The primers for amplification of *clpV* and *vgrG* were designed using Beacon Designer software (http://www.premierbiosoft.com/molecular beacons/) as part of the current study. The 16S rRNA-gene primers were as previously reported (Zhai et al., 2018). Primer sequences are shown in Table 2. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Strain were grown as described for the growth analysis on McConkey agar above as well as on LB agar under the same conditions. The analysis was performed to compare expression after growth at 41 °C and 37 °C. After incubation, bacteria were collected and immediately mixed with 1 ml RNA later (Ambion, Naerum, Denmark). Quality assurance and qPCR was performed as previously described (Moller et al., 2017). Four independent repeats were carried out.

2.8. Macrophage survival assay

Standard gentamycin protection assay was performed to determine survival of *S.* Gallinarum and *S.* Gallinarum ΔSPI19/T6SS mutant in HD11 macrophage-like cells, following the protocol described (Herrero-Fresno et al., 2018).

2.9. Intestinal loop assay

Intestinal loop experiments were performed as previously described (Aabo et al., 2000). The animals used were 16-week old Isa-Brown hens. Each loop was infected with either 5×10^7 bacteria from a single strain or a mixture of WT and the ΔSPI19/T6SS mutant. Inoculum was prepared from ON cultures as described for mouse experiments below. CFU per biopsy after 4 h of infection, the last 2 h with gentamycin to kill non-invading bacteria, was determined as described (Aabo et al., 2000). In competition experiments, the ratio between WT and mutant was determined as described under competitive challenge experiments below. Invasion of *S.* Typhimurium 4/74 and its Δ*invH* mutant was measured in parallel. The *invH* mutants showed the expected approximately 1 log reduction in invasion compared to the wild type strain.

2.10. Cytotoxicity towards macrophages

The ability of strains, with or without SPI19/T6SS, to interact and kill macrophages was determined using HD11 and BoMac cultured macrophage-like cells, as well as monocyte-derived primary macrophages from hens obtained following a previously reported method with some modifications (Wigley et al., 2002). Briefly, the heparinized hen blood was taken from the wing vein of salmonella-free Bovans Brown hens and then mixed with an equal volume PBS buffer. A 50 ml centrifuge tube containing 15 ml Ficoll-Paque Premium 1.084 solution (GE Healthcare) was carefully and slowly layered with 15 ml half-diluted blood on the top of the Ficoll solutions and then centrifuged at 1200 *g* for 40 min (Acceleration = 1, Brake = 0, horizontal centrifuge). The blood mononuclear cells were collected from the ring interface and then washed with DPBS buffer 3 times. The cell pellet was re-suspended and cultured in RPMI-1640 medium (Gibco) supplemented with 5% chicken serum (Sigma), 5% foetal bovine serum (Gibco), 25 μg/ml gentamycin and 200 μM L-glutamine at 37 °C with 5% CO2. The cell concentration was determined by counting using a haemocytometer. The cell culturing medium was changed every 24 h for the first three days to remove non-adherent cells. Phase contrast microscopy was used to monitor and record the cells population and morphology development. The microscopic analysis was undertaken for consecutive 7 days after seeding the cells into flask and plates.

Standard gentamycin protection assay was performed following the protocol described (Herrero-Fresno et al., 2018). The induced cell death was assessed by measuring the released cytosolic lactate dehydrogenase (LDH) in the supernatant as previously reported (Meunier et al., 2014), however, with some minor modifications. Briefly, at 20 h post infection, the supernatants including the infection and un-infected control groups were collected. The released LDH activities were then measured by using a colorimetric Cytotox 96 kit (Promega, Denmark). The percent cytotoxicity was calculated using the formula: % Cytotoxicity = (infected cell LDH release – uninfected cell LDH release)/ (maximum LDH release - uninfected cell LDH release) ×100, where the maximum LDH release was determined from total cell lysates. Cytotoxicity of *S.* Typhimurium 4/74 infected cells was measured in each experiment for comparison.

2.11. Animal challenge experiments

2.11.1. Challenge of mice and one-week old chicken for competitive indexing

Six-week-old female C57/BL6 mice were challenged with wild type and mutant strains of *S.* Dublin in a 50:50 ratio, essentially as described. Strains were given orally in a number of 1×10^6 of each bacterium per challenge and in a dose of 1×10^3 bacteria when administered intraperitoneally. One to two-week old Loman brown chicken (N = 4–6) were challenged with 2×10^9 of *S.* Gallinarum in oral challenge, and with 1×10^6 *S.* Gallinarum when intra-peritoneal challenge was performed (Herrero-Fresno et al., 2018; Schroll et al., 2014). Mice and

chicken were killed by cervical dislocation six days post challenge, and the ratio between mutant and wild type bacteria in spleens was determined based on determining the antimicrobial susceptibility of 200 colonies. The in vivo competition index (CI) was calculated as described (Wallrodt et al., 2013). Mice and chicken showing clear signs of being severely affected before the sixth days were immediately killed for animal welfare reasons, but otherwise treated as other animals.

2.11.2. Challenge of mice and chicken with single strains

Oral infection of mice with individual bacterial strains was performed, essentially as described for the competition experiments above. The only differences were that each mouse was infected with bacteria of just one strain, and the final bacterial load in the spleen was estimated by CFU counting on LB agar after homogenizing the spleen in five ml 0.9% NaCl.

2.11.3. Ethical statement

Animal experiments were carried out with permission from the Danish Experimental Animal Inspectorate to the senior author, license number: 2009/561–1675.

2.12. Statistics

GraphPad Prism 6 (GraphPad Software, Inc.) was used for statistical analysis. Prior to statistical analysis CFU counts were $\log_{10}$ transformed. The detection limit for bacteria in the spleen was 10^2 CFU, and for statistical comparisons, a $\log_{10}$ value of 2.0 was given to mice where no bacteria were retrieved from the sample, as previously described (Olsen et al., 1999). Outlier results were identified with Grubb's outlier test. Differences in CFU, cytotoxicity, and expression of genes at different temperatures, as well as competitive indexes, were analysed with two-tailed unpaired t-tests. When analysing differences between competitive indexes, the ratio of wild type to mutant strain in the input pool (challenge) was compared to the ratio between the same two strains in the output pool (spleen of animals at day 6), as suggested (Olsen et al., 2013).

3. Results

3.1. Mutant growth characterization

Wild type *S*. Dublin 2229, *S*. Gallinarum G9 and *S*. Gallinarum J91, ΔSPI19/T6SS and ΔSPI19 (J91) mutants of the same strains, and the complemented *S*. Dublin ΔSPI19/T6SS mutant did not differ with regard to growth in neither LB media (Supplementary material, Figure S2 – results for J91 not shown) nor M9 media (data not shown). The *S*. Gallinarum strains were also grown in LB media at 41 °C with no difference observed between strains (data not shown). The effect on growth from harbouring the plasmids, pCas1 and pSDubSPI19-1, which were used for complementation, was analysed in *S*. Typhimurium 4/74, where they did not affect growth (Supplementary material, Figure S2).

3.2. Co-culture of ΔSP19/T6SS mutants with strains of E. Coli

SPI6/T6SS in *S*. Typhimurium affects colonization in the intestine by killing intestinal bacteria (Sana et al., 2016). *S*. Dublin and in particular *S*. Gallinarum are not believed to depend on growth in the intestine for its long-term survival (Uzzau et al., 2000), and we hypothesized that the lack of SPI19/T6SS would not affect the outcome of competition experiments with strains of *E*. coli in these two serovars. Indeed, *S*. Gallinarum G9 ΔSP19/T6SS competed equally well as the *S*. Gallinarum G9 WT when co-cultured with *E*. coli strains (Fig. 1, bottom). Interestingly, the *S*. Gallinarum strains were outcompeted by strains of *E*. coli (irrespective of the presence of SPI19/TSS6). When grown as single strains on McConkey agar, but otherwise treated as in the assay, the wild type and mutant *S*. Gallinarum strains grew to
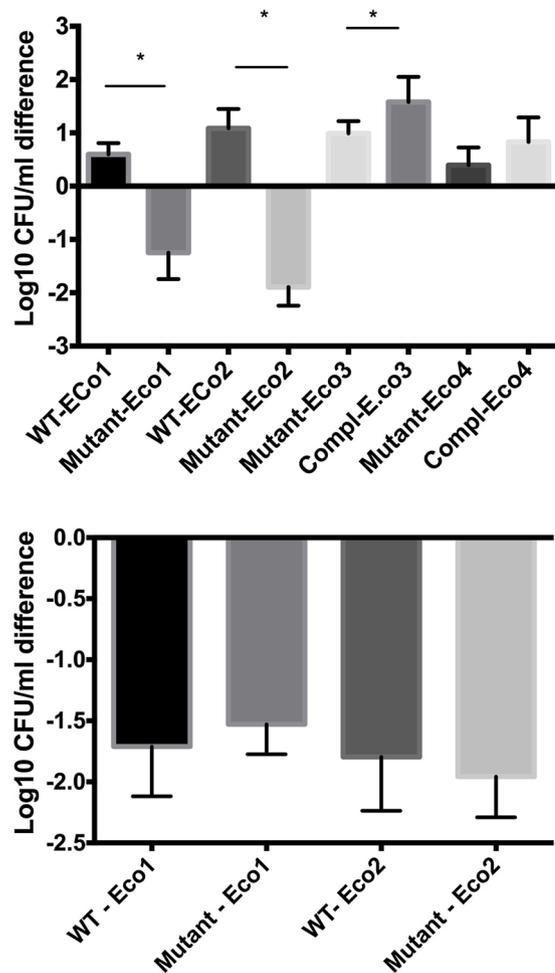

Fig. 1. Top: Competition between *S*. Dublin WT (WT) and *S*. Dublin ΔSPI19/T6SS (mutant) and commensal *E*. coli strains (Eco 1–4) and (bottom) competition between *S*. Gallinarum G9 (WT) and *S*. Gallinarum G9 ΔSPI19/T6SS (mutant) and commensal *E*. coli strains (Eco 1–2). Strains were seeded in equal numbers on the surface of McConkey agar and allowed to grow for 24 h, at which time the CFU of the two strains was determined. Results are the average of differences in CFU at 24 h based on 7–10 independent repeats.

approximately the same numbers (CFU 7.98 ± 0.21 $\log_{10}$), while strain of *E*. coli on average reached CFU 7.75 ± 0.25 $\log_{10}$. Contrary to our expectations, however, *S*. Dublin behaved as *S*. Typhimurium and was significantly affected by the lack of SPI19/T6SS. WT *S*. Dublin outgrew the *E*. coli strains while the mutants were outcompeted. This phenotype was first detected with the same two strains of *E*. coli as used for testing of *S*. Gallinarum. Next, the complemented strain was assayed in competition against two other *E*. coli strain, and complementation *in trans* was shown to increase fitness, however, in one experiment, the difference between mutant and complemented strain was not significant (Fig. 1, top). Plated alone, the WT, mutant and complemented mutant strains reached the same numbers (CFU 8.14 ± 0,18 Log10). Thus, SPI19/T6SS in *S*. Dublin may play a role during the intestinal phase of *S*. Dublin infection, as has previously been observed for *S*. Typhimurium (Sana et al., 2016).

3.3. Expression of SPI19/T6SS in S. Dublin and S. Gallinarum

Since *S*. Gallinarum normally infects chicken with at body temperature of 41 °C to 42 °C, the lack of advantage for *S*. Gallinarum having the T6SS when competing with strains of *E*. coli could be because the T6SS is only expressed at this high temperature, and not at 37 °C, the temperature where we performed the competitive growth

Table 3

The influence of growth media and growth temperature on expression[a] of *clpV* and *vgrG* of the SPI19 encoded T6SSs in *S.* Dublin and *S.* Gallinarum.

	clpV 41 °C versus 37 °C		*vgrR* 41 °C versus 37 °C	
	McConkey agar	LB agar	McConkey agar	LB agar
S. Dublin 2229 WT	3.41 ± 1.94 (p = 0.055)	0.80 ± 0.99 (p = 0.26)	3.66 ± 2.26 (p = 0.07)	0.97 ± 1.76 (p = 0.23)
S. Dublin ΔSPI19/T6SS complemented	0.38 ± 3.93 (p = 0.87)	0.30 ± 1.57 (p = 0.66)	−1.07 ± 4.56 (p = 0.71)	−0.74 ± 1.33 (p = 0.41)
S. Gallinarum G9 WT	1.31 ± 1.59 (p = 0.25)	2.47 ± 1.57 (p = 0.07)	0.79 ± 1.69 (p = 0.48)	2.59 ± 1.60 (p = 0.07)

a: Expression is expressed as $\log_2$ fold change at 41 °C compared to expression of the same gene at 37 °C. p-values for the nil-hypothesis that the differences are non-significant are shown in brackets.

experiments. To rule out this, we performed expression analysis of two core genes of the T6SS, *clpV* and *vgrG* after growth at 37 °C and 41 °C on McConkey and LB-agar. Results are shown in Table 3. Expression increased slightly in *S.* Gallinarum with increased temperature when grown on LB agar, however, not significantly, while *clpV* was slightly up-regulated and *vgrG* slightly down regulated (also non-significant) after growth on McConkey agar, which was the media used in the growth competition assay. Expression in *S.* Dublin went up with temperature after growth on McConkey agar, while it was unchanged after growth on LB-agar. The expression in the complemented mutant of *S.* Dublin generally decreased with temperature, suggesting a difference in regulation in this strain compared to the WT *S.* Dublin.

3.4. Survival in HD11 macrophages and cytotoxicity towards HD11 and primary macrophages

The phenotypes reported so far for SPI19/T6SS in *S.* Gallinarum is lower CFU at intestinal and systemic sites following oral infection (Blondel et al., 2009) and decreased survival inside macrophages at 20 h post infection. The authors (Blondel et al., 2013) produced the results of macrophage survival using HD11 cultured macrophages grown at 37° C and showed that the decreased survival was not related to changed cytotoxicity (Blondel et al., 2013). No studies have so far been published trying to confirm this important observation. To be able to compare directly to this study, we used a mutant of strain G9, in which the whole genome island had been deleted. Comparison was made to a rifampicin resistance G9 wild type. Contrary to Blondel et al. (Blondel et al., 2013), we did not observe significant difference in survival rate for the *S.* Gallinarum G9 ΔSPI19 mutant compared to the WT[rif] in HD11 cells 24 h post challenge (Fig. 2). The study was extended to 48 h. Even at this late time point, no significant difference was present (Supplementary material, Fig. S3). Using primary macrophages
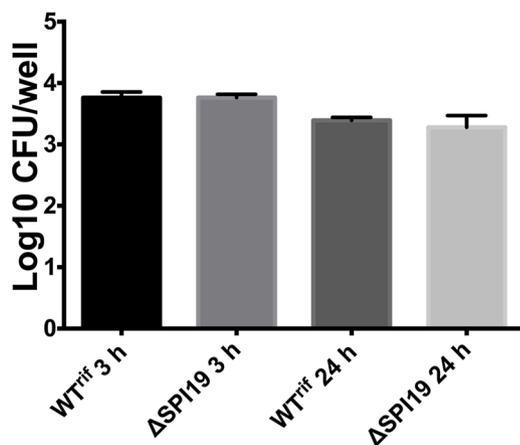

Fig. 2. CFU counts of *S.* Gallinarum G9[rif] and *S.* Gallinarum G9 ΔSPI19 mutant in HD11 macrophage cells. Results shown are from four independent experiments.

from hens, we observed that the WT strain was significantly more cytotoxic than the SPI19/T6SS mutant (Table 4). In cultured HD11 and BoMac macrophages, the ΔSPI19/T6SS mutant was also less cytotoxic than the WT strains, however, the differences in cytotoxicity between the wild type and mutant strain were not significant (Table 4). Thus, the T6SS encoded in SPI19 of *S.* Gallinarum appeared to affect cytotoxicity towards primary macrophages. *S.* Dublin was assayed in a similar way. There was no significant difference between *S.* Dublin 2229 and *S.* Dublin ΔSPI19/T6SS and the complemented mutant in this testing, and indeed in HD11 cells, the mutant was more cytotoxic than the WT strain, however not significantly (p = 0.054) (Table 4). The cytotoxicity of the *S.* Typhimurium wild type strains was included for comparison. This strain was found to vary considerably in cytotoxicity depending on the origin of the macrophages, and in particular, it was found to be very toxic to HD11 cells.

3.5. The role of SPI19/T6SS in infection with S. Dublin and S. Gallinarum

Our results above suggested a possible role of SPI19/T6SS in the intestine during *S.* Dublin infection, while the SPI19/T6SS in *S.* Gallinarum was more likely to affect systemic infection due to macrophage cytotoxicity. Animal challenge experiments were carried out to further investigate this. First, *S.* Dublin ΔSPI19/T6SS was tested for virulence in mouse in competition with the *S.* Dublin 2229 wild type using both oral and intraperitoneal routes of infection. The Competitive Index (CI) for the intraperitoneal administration was not significantly different from WT virulence (p = 0.82), while the CI for the oral challenge was significantly lower (p = 0.02) (Table 5). These results showed a difference in virulence depending on the infection route. In order to confirm the role of SPI19/T6SS in oral infection with *S.* Dublin, mice challenge experiments were repeated, but this time using single strain infections to rule out the possibility of interaction between WT and the SPI19/T6SS deleted strain. At the termination of the experiment at day 6, mice infected orally with the *S.* Dublin ΔSPI19/T6SS mutant had significantly (p < 0.004) less CFU in the spleen than the wild type strain. The attenuation could be complemented by providing the missing part of the T6SS *in trans*, however, not to the full virulence of the WT strain (Table 5). In order to understand why the complementation did not restore virulence fully, we performed a competitive challenge with *S.* Dublin 2229 and *S.* Dublin 2229 carrying the plasmid pCAS1, which was the backbone of the complementation plasmid. The CI was 0.62 ± 0.12, indicating a slight fitness burden by carrying this plasmid. Taken together, the results showed a significant role for *S.* Dublin 2229 SPI19 in oral infections, but not in intraperitoneal infection of mice.

Blondel et al. (2009) demonstrated that *S.* Gallinarum ΔSPI19/T6SS colonized the intestine in lower numbers than wild type and that lower numbers of bacteria were found at systemic sites following oral challenge. We used spleen counts as end-points following challenge of one-week old chicken with *S.* Gallinarum ΔSPI19/T6SS in competition with the wild type G9 strain. The *S.* Gallinarum G9 ΔSPI19/T6SS mutant was less virulent when given orally compared to when given intraperitoneally, but the

Table 4

Cytotoxicity of strains towards cultured HD11 and BoMac macrophages, and primary macrophages from hens.

Strain/ macrophage	S. Ga G9	S. Ga G9 ΔSPI19/T6SS	S. Du 2229	S. Du 2229 ΔSPI19/T6SS	S. Du 2229 ΔSPI19/T6SS complemented	S. Tm 4/74
HD11	8.13 ± 2.14 (n = 3)	3.67 ± 1.75[NS] (n = 3)	7.51 ± 0.81 (n = 3)	17.70 ± 3.70[NS] (n = 3)	17.70 ± 5.23[NS]	51.52 ± 9.17 (n = 3)
BoMac	10.29 ± 1.88 (n = 4)	7.04 ± 2.43[NS] (n = 4)	4.30 ± 1.45 (n = 4)	4.24 ± 1.60[NS] (n = 4)	3.97 ± 2.52[NS]	0.73 ± 1.06 n = 4)
Primary macrophages	4.84 ± 0.40 n = 3	1.26 ± 1.16[*] n = 3	2.66 ± 0.45 n = 3	1.42 ± 0.94[NS] n = 3	0.60 ± 2.01[NS] n = 3	4.25 ± 0.82 n = 3

Results are means ± SEM. *S.* Ga = *S.* Gallinarum, *S.* Du = *S.* Dublin, *S.* Tm = *S.* Typhimurium. * = Significantly different from WT 0.05 > p > 0.01. NS = not significantly different from wild type.

attenuation in oral challenge was not significant, i.e. the proportion of mutant in the input and output pool was not significantly different (p = 0.46) (Table 5). Since this result contrasted previously published results (Blondel et al., 2010), we confirmed it by making an independent mutation in the same strain covering the full TSS6 and by transferring the mutation to another wild type strain (J91). These two mutants also did not differ significantly from wild type virulence in competitive indexes following oral challenge (data not shown). To further dissect this result, we performed loop-test to investigate whether wild type and mutant strain invaded the intestine in equal numbers, despite the mutant being reported to be less good a colonizing in the intestine. The mutant and the wild type strains did not differ with respect to invasion capability (Fig. 3).

4. Discussion

Most studies of the role of T6SSs in virulence of *Salmonella* have been concerned with *S.* Typhimurium and *S.* Gallinarum. Results produced with *S.* Gallinarum strain 287/91 indicated a role of SPI19/T6SS in intestinal colonization (Blondel et al., 2010) and in long term survival inside cultured macrophages (Blondel et al., 2013). The intestinal effect was reported not to be related to increased ability to kill other bacteria, and the macrophage survival did not relate to decreased cytotoxicity towards the macrophages. This suggested a role of T6SS which is different from the current model of T6SS as one of killing other cells. In the current study, we failed to reproduce the observation with regard to differences in survival in macrophages at late time point (24 h and 48 h), using the same cell line (HD11) as in the original report, and we showed that the WT and mutant did not differ with respect to cytotoxicity towards HD11 cells and in the ability to kill *E. coli*. However, in primary macrophages from hens, which is a more realistic model for studies of interaction between *S.* Gallinarum and macrophages, we saw a modest, yet significant reduction in cytotoxicity when the SPI19 encoded T6SS was deleted. The same tendency was observed using HD11and BoMac macrophages, yet with non-significant differences to the wilt type strain. We used a different strain (G9) than Blondel et al. (Blondel et al., 2013) used in their studies, and we cannot rule out that this is important for the differences observed in survival in HD11 between the two investigations.

Interestingly, expression of two core genes of the T6SS in *S.* Gallinarum was not significantly increased at 41 °C, corresponding to
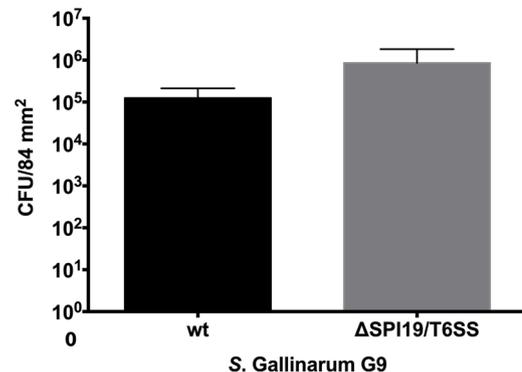

Fig. 3. Invasion of *S.* Gallinarum G9 (WT) and *S.* Gallinarum G9 ΔSP19/T6SS (GGI) mutant in the small intestine of hens. Bacteria were injected into intestinal loops and bacteria were harvested by taking biopsy of the intestinal wall 4 h post injection (the last two hours with gentamycin in the loop to kill extracellular bacteria). Results shown for the WT is average of two loops and results shown for the mutant are average of four loops.

the body temperature of the hen, compared to 37 °C. This may indicate that the system is not fine-tuned to expression at systemic sites, and further studies of expression in the host is indicated to determine this.

When assessing the importance of SPI19/T6SS in virulence, we used bacterial counts at systemic sites as the end point of infection, and did not determine CFU at intestinal sites, as included in the investigation by Blondel et al. (Blondel et al., 2010). *S. Gallinarum* is a host restricted serovar and it causes systemic disease. Inflammation in the intestine is not considered important for this serovar (Uzzau et al., 2000). In support of this, the genome contains a high degree of pseudogene formation in genes of importance for utilization of nutrients in the inflamed intestine (Langridge et al., 2015), and SPI-1, which is essential for induction of inflammation in the intestine (see (Herrero-Fresno and Olsen, 2018) for a recent review) is dispensable for infection in this serovar (Jones et al., 2001). Hence, the real importance of SPI19/T6SS in *S.* Gallinarum should be reflected at the systemic end-point. Our study showed lower numbers of bacteria in the spleen of the *S.* Gallinarum G9 ΔSPI19/T6SS mutant 5–6 days post infection compared to the WT G9 wild type strain, but the reduction in virulence was not significant. We showed this for both a ΔSPI19/T6SS and a ΔSPI19

Table 5

Virulence testing of ΔSPI19/T6SS mutants of *S.* Dublin[a] and *S.* Gallinarum[b] in mice and chicken, respectively.

	WT S. Dublin 2229	S. Dublin ΔSPI19/T6SS	S. Dublin ΔSPI19/T6SS complemented	WT S. Gallinarum G9	S. Gallinarum ΔSPI19/T6SS
CI[c] oral challenge	NR[e]	0.40 ± 0.21 (p = 0.02)	–	NR	0.79 ± 0.21[NS]
CFU in spleen day 6 after oral challenge	6.22 ± 0.70	2.00 ± 0.00 (p < 0.001)	4.22 ± 1.28[NS]	–	–
CI[c] ip[d] challenge	NR	0.85 ± 0.16 (p = 0.82)	–	NR	1.05 ± 0.25[NS]

a: Strain of *S.* Dublin were tested for virulence in mice, b: Strain of *S.* Gallinarum were tested for virulence in one week old chicken. c: Competitive virulence index. d: intra-peritoneal challenge. e: Wild type virulence is per definition 1.00 in competitive indexing. NS: Not significantly different from WT virulence.

mutant of strain G9 and a ΔSPI19 mutant of another strain J91. Host specificity of *S.* Gallinarum to avian hosts is not expressed at the intestinal level, since this bacterium is not superior to other serovars in invading in the chicken intestine (Chadfield et al., 2003). According to our investigation, the *S.* Gallinarum G9 SPI19/T6SS does not play a role in invasion (*per se*). In *S.* Typhimurium, the SPI6/T6SS mutant does not differ from the wild type in mouse virulence, when challenge bypass the intestine (Sana et al., 2016). In the current study, we found similar results for *S.* Gallinarum, i.e., the competitive index of the *S.* Gallinarum ΔSPI19/T6SS mutant was approximately 1.00 following IP challenge.

Results of the current study showed a clear role in oral pathogenicity for the T6SS encoded from SPI19 in S. Dublin 2229. From in vitro experiments, the role appeared to be killing of competing bacteria, which corresponds to what has been observed for the SPI6 encoded T6SS of S. Typhimurium (Sana et al., 2016), however, only in vivo data with CFU counts from the intestine of infected animals would allow a firm conclusion in this matter. The result is in contrast to previous reporting, where only the T6SS of *S.* Dublin encoded in SPI6 was shown to affect virulence, while the SPI19/T6SS was dispensable (Pezoa et al., 2014). The T6SS consists of 13 highly conserved T6SS core proteins each of which belongs to a specific COG (Boyer et al., 2009). The deletion made by us in SPI19 of S. Dublin was internal to the deletion previously reported (SeD_A1217 to SedA1236 as opposed to SeD_A1212 to SeD_1243). Our deletion targeted the core T6SS genes, while (Pezoa et al., 2014) knocked out the entire genome island. It is however, difficult to see how this could cause the discrepancy between the two studies. There may be important strain differences. *S.* Dublin strain CT_02021853 used by Pezoa et al. (Pezoa et al., 2014) was capable of causing systemic infection in chicken, a feature we have never seen with strains of *S.* Dublin, and which is not present in *S.* Dublin strain 2229 used by us. This strain invades in the intestine of hens to the same extend as *S.* Gallinarum G9, but it cannot be re-isolated from systemic sites 5 days post challenge of one-week old chicken (Chadfield et al., 2003). Further studies are needed to understand the difference between the two studies. Like it has been observed for SPI6/T6SS in S. Typhimurium (Sana et al., 2016), the SPI19/T6SS in *S.* Dublin did not affect the outcome of infection, where the gut was by-passed. In support of this, we saw no significant difference in interaction with macrophages between wild type and mutant *S.* Dublin. Importantly, both the study performed by Pezoa et al. (Pezoa et al., 2014) and by us have used mice as infection model in studies of *S.* Dublin. It would be interesting to use the natural host, cattle, to see if the SPI19/T6SS plays the same role in infection of this animal as in mice.

In conclusion, we have demonstrated a role of SPI19/T6SS for *S.* Dublin oral but not systemic infection in mice, while we could not demonstrate a clear role in virulence for the same system in *S.* Gallinarum, irrespective of challenge route. Uzzau et al. {Uzzau, 2000 #2} grouped *S.* Dublin and *S.* Choleraesuis as host adapted serotypes, because they are capable of infecting more than one host, yet are most commonly isolated from just one host. From the results of the current study, it appears that the host adapted *S.* Dublin depends on growth in the intestine and has developed specific weapons (T6SS) to compete with the intestinal flora. Contrary to this, *S.* Gallinarum was grouped as strictly host specific {Uzzau, 2000 #2}, and one would expect the SPI19/T6SS to have a role during systemic infection. Further studies are needed to understand why the SPI19/T6SS is so well conserved in *S.* Gallinarum, as demonstrated by genome analysis (Blondel et al., 2009), considering the lack of attenuation we observed for the SPI19/T6SS in both oral and intra-peritoneal challenge of chicken.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2019.01.006.

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